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(57) Abstract Methods to negatively regulate LFA-1 binding to an ICAM that binds LFA-1 are provided, in addition to a novel regulatory binding site on LFA-1.			

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LFA-1 REGULATORY BINDING SITE AND USES THEREOF

BACKGROUND

The leukocyte function-associated antigen (LFA-1, CD11a/CD18) is a leukocyte-specific β_2 integrin that participates in cell/cell adhesion. Binding activity of LFA-1 is essential to leukocyte extravasation from circulation to a site of injury in an inflammatory response. Three principle ligands are known to bind LFA-1, ICAM-1, ICAM-2, and ICAM-3. These are intercellular adhesion molecules that play an important role in localizing leukocyte adhesion to endothelial cells at a site of injury. ICAM-4 and ICAM-5 have also been reported to bind LFA-1. Most leukocytes constitutively express LFA-1, but ligand binding requires activation believed to induce a conformational change and to increase avidity ligand binding. For example, ICAM-1 is normally expressed at low levels on the endothelium. However, injury-induced inflammatory mediators promote enhanced surface expression in cells at the site of the injury which, in turn, promotes localized leukocyte adhesion through binding to activated LFA-1.

The structure of LFA-1 includes distinct intracellular and extracellular domains that are believed to participate in and/or regulate ICAM binding. Of particular interest is a region in the α_L chain of approximately 200 amino acids, designated the I domain, that is found in all β_2 integrins, as well as many other proteins. Evidence suggests that the I domain is essential to LFA-1 binding to ICAM-1 and 3. For example, anti-LFA-1 blocking monoclonal antibodies have been mapped to epitopes within the I domain. In addition, recombinant I domain polypeptide fragments have been shown to inhibit integrin-mediated adhesion and bind ICAM-1. Within the I domain of LFA-1 (and other proteins) is a single metal ion dependent adhesion site (MIDAS) that preferentially binds manganese or magnesium ions. Binding of either cation is required for ligand interaction and is believed to induce conformational changes in LFA-1 necessary for binding. Cation binding may therefore be a regulatory mechanism that responds to changes in the extracellular leukocyte environment. This hypothesis is supported by the observation that calcium ion binding actually inhibits LFA-1 interaction with ICAM-1. Indeed, it has been

proposed that an inactive LFA-1 conformation results from calcium binding, and that replacement of the calcium ion with a manganese or magnesium ion is a step required for LFA-1 activation [Griggs, *et al.*, *J. Biol. Chem.* 273:22113-22119 (1998)]. Other factors have also been shown to induce LFA-1 activation, including T cell receptor engagement, cytokine stimulation, and *in vitro* PMA stimulation.

In practical terms, the identification of LFA-1/ICAM binding sites provides targets to modulate leukocyte inflammatory responses. Numerous antibodies have been isolated that are capable of inducing LFA-1 activation [see, for example, Landis, *et al.*, *J. Cell Biol.* 120:1519-1527 (1993)] or that are capable of preventing ICAM-1 interaction [see for example, Randi and Hogg, *J. Biol. Chem.* 269:12395-12398 (1994)]. The previous identification of anti-LFA-1 activating antibodies that recognize multiple and distinct extracellular epitopes suggests the existence of more than one regulatory region, presumably independent of cytoplasmic signaling. Localization of LFA-1 sites that bind ICAM-1 has been investigated through use of chimeric LFA-1 α subunit proteins comprising human and murine components [Huang and Springer, *J. Biol. Chem.* 270:19008-19016 (1995)]. Studies have indicated that residues that coordinate cation binding and residues proximal to the site are essential for binding ICAM-1 at a relatively flat interface. More precise delineation of the extracellular regulatory region(s) and the contact points for ICAM-1 binding will permit design of efficient modulators.

Thus there exists a need in the art to precisely identify regulatory regions for proteins that participate in inflammatory responses, and in particular LFA-1 and ICAMs that bind LFA-1. Determining the tertiary (or quaternary) structure of a protein can identify potential regulatory regions to permit the rational design of biologically compatible small molecules for therapeutic and prophylactic intervention for inflammatory disorders. There further exists a need in the art to identify compounds that can inhibit LFA-1 binding to ICAMs that can be used in the treatment of inflammatory disorders.

SUMMARY OF THE INVENTION

The present invention provides methods for identifying a negative regulator of LFA-1 binding to a natural ligand that competes for binding to LFA-1 with ICAM-1 or ICAM-3 comprising the steps of (i) contacting LFA-1, or a ligand binding fragment thereof, and a ligand that binds LFA-1, or an LFA-1-binding fragment thereof, in the presence and absence of a test compound under conditions that allow binding of LFA-1 to the ligand (ii) identifying as a negative regulator the compound which decreases LFA-1 binding to the ligand and which binds LFA-1 α_L polypeptide at a site presenting a diaryl sulfide binding conformation defined by Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹, and Ile³⁰⁶ of human LFA-1 as set out in SEQ ID NO: 2, which provides the amino acid sequence for mature (*i.e.*, without the leader sequence) LFA-1. "Natural ligand" refers to any biological compound that binds LFA-1. The term "negative regulator" refers to a compound that decreases ICAM binding to LFA-1, but does not directly compete with the ICAM for LFA-1 binding. A negative regulator may be an allosteric inhibitor or a compound that modulates the activation state of LFA-1. In a preferred method, the negative regulator is a diaryl sulfide. In a preferred embodiment, the natural ligand is an ICAM. Most preferably, the ICAM is ICAM-1 or ICAM-3.

In another aspect, the invention provides methods for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising the steps of (i) contacting LFA-1, or a ligand binding fragment thereof, and a natural ligand that binds LFA-1, or an LFA-1-binding fragment thereof, in the presence and absence of a test compound under conditions that allow binding of LFA-1 to the ligand, (ii) identifying as a negative regulator the compound which decreases LFA-1 binding to the ligand and which competes with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide for binding to LFA-1 α_L polypeptide. In a preferred method, the negative regulator is a diaryl sulfide. Preferably, the ligand is an ICAM. Most preferably, the ICAM is ICAM-1 or ICAM-3.

The invention also provides screening methods for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising

the steps of (i) contacting LFA-1, or a ligand binding fragment thereof, with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]-sulfide in the presence and absence of a compound, and (ii) identifying the compound as a putative negative regulator wherein the compound competes with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]-sulfide for binding to LFA-1 α_L polypeptide. In a preferred method, the negative regulator is a diaryl sulfide.

The invention also provides pharmaceutical compositions comprising a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 identified by a method of the invention. The invention further provides use of a negative regulator identified by a method of the invention in the production of a medicament to ameliorate pathologies arising from LFA-1 binding to an ICAM that binds LFA-1.

The invention further provides methods for inhibiting LFA-1 binding to a natural ligand that binds LFA-1 comprising the step of contacting LFA-1, or a ligand binding fragment thereof, with a negative regulator compound; said negative regulator binding the LFA-1 α_L polypeptide, or a fragment thereof, at a site selected from the group consisting of a conformation that binds a diaryl sulfide, a site defined by Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹, and Ile³⁰⁶ of human LFA-1 α_L polypeptide, and an LFA-1 domain that binds (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide. In a preferred method, the negative regulator is a diaryl sulfide. In one embodiment, methods of the invention include use of cells expressing either LFA-1 or the ligand. In methods wherein one of the binding partners is expressed in a cell, the other binding partner is either purified and isolated, in a fluid sample (purified, partially purified, or crude) taken from an individual, or in a cell lysate. The invention also comprehends methods wherein both LFA-1 and the ICAM are expressed in cells. The LFA-1 and ligand binding partners may be expressed on the same cell type or different cell types.

The invention also provides methods to inhibit leukocyte adhesion to endothelial cells comprising the step of contacting said leukocyte with a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1, said negative regulator binding an LFA-1 regulatory site selected from the group consisting of a site

that binds a diaryl sulfide, a site defined by Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹, and Ile³⁰⁶ of human LFA-1 α_L polypeptide, and an LFA-1 domain that binds (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide. *In vivo* and *in vitro* methods are contemplated. In a presently preferred embodiment, the negative regulator of the methods is a diaryl sulfide and the regulatory binding is reversible.

The invention also provides methods to ameliorate a pathology arising from LFA-1 binding to a natural ligand that binds LFA-1 comprising the step of administering to an individual in need thereof a negative regulator of LFA-1 binding to the ligand in an amount effective to inhibit LFA-1 binding to the ligand, said negative regulator binding to an LFA-1 regulatory site selected from the group consisting of a site that binds a diaryl sulfide, a site defined by Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹, and Ile³⁰⁶ of human LFA-1 and an LFA-1 domain that binds compound (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]-sulfide.

The invention also provides LFA-1 α_L polypeptides and fragments thereof comprising a regulatory binding site presenting a diaryl sulfide binding conformation. In one aspect, the LFA-1 polypeptide fragment comprises the α_L polypeptide I domain sequence. Preferably, the LFA-1 polypeptide contains less than all amino acids in the α polypeptide I domain. The invention also provides mutant LFA-1 polypeptides wherein amino acid residues in the wild type α_L polypeptide regulatory site are substituted with non-naturally occurring (*i.e.*, residues not found in the same position in the wild type molecule) amino acid residues. Preferred mutant regulatory sites exhibit modified affinity and/or avidity for an ICAM, both in the presence and absence of an inducing agent (*e.g.*, the monoclonal antibody 240Q described below which induces LFA-1 into an activated state required for ICAM binding). Presently preferred mutants include (i) those demonstrating wild type levels of ICAM-1 binding with or without monoclonal antibody 240Q induction, exemplified mutations having one or more of the single amino acid changes Val¹⁵⁷-Ala, Glu²¹⁸-Ala, Thr²³¹-Ala, Lys²⁸⁰-Ala, and Lys²⁹⁴-Ala, (ii) mutants that support greater than wild type levels of binding without induction and wild type levels

with induction, exemplified by mutations having one or more of the single amino acid changes Ile²³⁵-Ala, Ile²⁵⁵-Ala, Ser²⁸³-Ala, Glu²⁸⁴-Ala, Glu³⁰¹-Ala, and Ile³⁰⁶-Ala, (iii) mutants with decreased levels of ICAM-1 binding relative to wild type binding in the absence of induction, but wild-type levels with antibody 240Q induction, exemplified by mutants having one or more of the substitutions Lys¹⁶⁰-Ala, Lys²³²-Ala, Asp²⁵³-Ala, Lys²⁸⁷-Ala, Gln³⁰³-Ala, Lys³⁰⁴-Ala, and Lys³⁰⁵-Ala, and (iv) mutants demonstrating severely decreased levels or no ICAM-1 binding with or without induction, exemplified by a mutant with the single substitution Tyr³⁰⁷-Ala.

The invention also provides an LFA-1-activating monoclonal antibody secreted by a hybridoma designated 240Q, mailed on March 30, 1999 to, and received on March 31, 1999 by the American Type Culture Collection, 10861 University Blvd., Manassas, VA 20010-2209, and assigned Accession No: HB-12692.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel *in vivo* and *in vitro* methods for negatively, and preferably reversibly, regulating LFA-1 binding to a natural ligand that binds LFA-1 involving use of compounds which bind LFA-1 at a regulatory domain located remote from the ligand binding site. The LFA-1 regulatory site presents a conformation that binds a substituted diaryl sulfide. The binding site is defined by human LFA-1 amino acid residues Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹ and Ile³⁰⁶. Alternatively, the site is defined by amino acid residues Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹, Ile³⁰⁶, Leu³⁰², Tyr²⁵⁷, Leu¹³², Val²³³, Val¹³⁰, and Tyr¹⁶⁶. In still another alternative, the binding site is defined by amino acid residues Lys²⁸⁷, Leu²⁹⁸, Ile²⁵⁹, Leu³⁰², Ile²³⁵, Val¹⁵⁷, Tyr²⁵⁷, Lys³⁰⁵, Leu¹⁶¹, Leu¹³², Val²³³, Ile²⁵⁵, Val¹³⁰, Tyr¹⁶⁶, Ile³⁰⁶, Phe¹³⁴, Phe¹⁶⁸, Phe¹⁵³, Tyr³⁰⁷, Val³⁰⁸, Ile³⁰⁹, Thr²³¹, Glu²⁸⁴, Phe²⁸⁵, Glu³⁰¹, Met¹⁵⁴, Ile²³⁷, Ile¹⁵⁰, and Leu²⁹⁵. Preferably, the ligand is an ICAM. Most preferably, the ICAM is ICAM-1 or ICAM-3.

In a presently preferred embodiment, reversible negative regulation (*i.e.*, reversible inhibition) of LFA-1 binding to ligand ICAM is provided by substituted diaryl sulfide compounds which bind LFA-1 at the aforementioned

regulatory domain and/or compounds that competitively inhibit diaryl sulfide binding to said domain.

In one aspect, methods of the invention are carried out using LFA-1 and a binding partner protein, such as ICAM-1, which are recombinant, purified from natural sources, or synthetic. In a preferred method of the invention, the LFA-1 and ICAM binding partner proteins are recombinant. The binding partner proteins may be holoproteins (*e.g.*, including both α and β chains of LFA-1), protein subunits (*e.g.*, the isolated LFA-1 α polypeptide chain), or fragments thereof, including, for example, extracellular domains of either LFA-1 or the ICAM, I domain fragments of LFA-1, less than complete I domain fragments of LFA-1, and/or less than a complete extracellular domain of the ICAM.

In another aspect, the invention provides methods wherein either LFA-1, the ligand, or both are expressed in a cell. When one or both binding partner proteins are expressed in a cell, the cell can be one that expresses an endogenous polynucleotide encoding LFA-1 or the ligand, or a host cell transformed and transfected with a heterologous polynucleotide encoding LFA-1 or the ligand and grown under conditions appropriate to permit expression of LFA-1 or the ligand on the cell surface. Regardless of whether cells of the methods express endogenous or heterologous polynucleotides encoding LFA-1 or the ligand, transcription of the polynucleotide can be directed by either endogenous or heterologous transcriptional control elements. For example, endogenous control elements can be purified from a desired host cell and ligated in an operative position relative to the LFA-1 or the ligand-encoding polynucleotide. Alternatively, a cell expressing endogenous LFA-1 or the ligand can be modified, for example through homologous recombination, to provide the LFA-1 or ligand polynucleotide with one or more transcriptional control elements that modify wild type levels of proteins expression. In assays involving cells expressing endogenous LFA-1 and ligand, preferred cells are leukocytes, *i.e.*, lymphocytes, monocytes, and granulocytes (*e.g.*, neutrophils), and endothelial cells.

In another aspect, the invention embraces methods to inhibit leukocyte adhesion to endothelial cells associated with LFA-1, expressed on leukocytes, binding to an ICAM that binds LFA-1, expressed on endothelial cells. Leukocyte adhesion to

endothelium is characteristic of an inflammatory response arising from release of cell mediators at an injury site. By providing methods to inhibit leukocyte adhesion to endothelial cells, the invention also comprehends methods to inhibit an inflammatory response associated with LFA-1 binding to a natural ligand that binds LFA-1.

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Therapeutic Methods

To the extent that leukocyte adhesion to endothelial cells gives rise to a pathological disorder, the invention provides methods to ameliorate pathologies associated with accumulation of leukocytes resulting from LFA-1 binding to an ICAM that binds LFA-1, comprising the step of administering to an individual in need thereof an amount of an inhibitor of LFA-1 binding to the ICAM effective to inhibit LFA-1 binding to the ICAM, said inhibitor binding to LFA-1 at a site presented by amino acid residues Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹ and Ile³⁰⁶. Exemplary medical conditions include, without limitation, inflammatory diseases, autoimmune diseases, 15 reperfusion injury, myocardial infarction, stroke, hemorrhagic shock, organ transplant, and the like. Methods of the invention provide for amelioration of a variety of pathologies, including, for example, but not limited to adult respiratory distress syndrome, multiple organ injury syndrome secondary to septicemia, multiple organ injury secondary to trauma, reperfusion injury of tissue, acute glomerulonephritis, 20 reactive arthritis, dermatosis with acute inflammatory components, stroke, thermal injury, Crohn's disease, necrotizing enterocolitis, granulocyte transfusion associated syndrome, cytokine induced toxicity, and T cell mediated diseases.

Inflammatory cell activation and excessive or unregulated cytokine (e.g., TNF α and IL-1 β) production are also implicated in disorders such as rheumatoid 25 arthritis, osteoarthritis, gouty arthritis, spondylitis, thyroid associated ophthalmopathy, Behcet disease, sepsis, septic shock, endotoxic shock, gram negative sepsis, gram positive sepsis, toxic shock syndrome, asthma, chronic bronchitis, allergic respiratory distress syndrome, chronic pulmonary inflammatory disease, such as chronic obstructive pulmonary disease, silicosis, pulmonary sarcoidosis, reperfusion injury of 30 the myocardium, brain, and extremities, fibrosis, cystic fibrosis, keloid formation, scar formation, atherosclerosis, transplant rejection disorders, such as graft vs. host

reaction and allograft rejection, chronic glomerulonephritis, lupus, inflammatory bowel disease, such as ulcerative colitis, proliferative lymphocyte diseases, such as leukemia, and inflammatory dermatoses, such as atopic dermatitis, psoriasis, urticaria, and uveitis.

5 Other conditions characterized by elevated cytokine levels include brain injury due to moderate trauma (see *J. Neurotrauma*, 12, pp. 1035-1043 (1995); *J. Clin. Invest.*, 91, pp. 1421-1428 (1993)), cardiomyopathies, such as congestive heart failure (see *Circulation*, 97, pp. 1340-1341 (1998)), cachexia, cachexia secondary to infection or malignancy, cachexia secondary to acquired immune deficiency syndrome (AIDS), ARC (AIDS related complex), fever myalgias due to
10 infection, cerebral malaria, osteoporosis and bone resorption diseases, keloid formation, scar tissue formation, and pyrexia.

 The ability of the negative regulators of the invention to treat arthritis can be demonstrated in a murine collagen-induced arthritis model [Kakimoto, *et al. Immunol.* 142:326-337 (1992)], in a rat collagen-induced arthritis model [Knoerzer, *et al.*, *Toxicol Pathol.* 25:13-19 (1997)], in a rat adjuvant arthritis model [Halloran, *et al.*, *Arthritis Rheum* 39:810-819 (1996)], in a rat streptococcal cell wall-induced arthritis model [Schimmer, *et al.*, *J. Immunol.* 160:1466-1477 (1998)], or in a SCID-
15 mouse human rheumatoid arthritis model [Oppenheimer-Marks, *et al.*, *J. Clin. Invest* 101:1261-1272 (1998)].
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 The ability of the negative regulators to treat Lyme arthritis can be demonstrated according to the method of Gross, *et al.*, *Science*, 218:703-706, (1998).

 The ability of the negative regulators to treat asthma can be demonstrated in a murine allergic asthma model according to the method of Wegner,
25 *et al.*, *Science*, 247:456-459, (1990), or in a murine non-allergic asthma model according to the method of Bloemen, *et al.*, *Am. J. Respir. Crit. Care Med.* 153:521-529 (1996).

 The ability of the negative regulators to treat inflammatory lung injury can be demonstrated in a murine oxygen-induced lung injury model according to the
30 method of Wegner, *et al.*, *Lung*, 170:267-279, (1992), in a murine immune complex-induced lung injury model according to the method of Mulligan, *et al.*, *J. Immunol.*,

154:1350-1363, (1995), or in a murine acid-induced lung injury model according to the method of Nagase, *et al.*, *Am. J. Respir. Crit. Care Med.*, 154:504-510, (1996).

5 The ability of the negative regulators to treat inflammatory bowel disease can be demonstrated in a murine chemical-induced colitis model according to the method of Bennett, *et al.*, *J. Pharmacol. Exp. Ther.*, 280:988-1000, (1997).

The ability of the negative regulators to treat autoimmune diabetes can be demonstrated in an NOD mouse model according to the method of Hasagawa, *et al.*, *Int. Immunol.* 6:831-838 (1994), or in a murine streptozotocin-induced diabetes model according to the method of Herrold, *et al.*, *Cell Immunol.* 157:489-500, (1994).

10 The ability of the negative regulators to treat inflammatory liver injury can be demonstrated in a murine liver injury model according to the method of Tanaka, *et al.*, *J. Immunol.*, 151:5088-5095, (1993).

The ability of the negative regulators to treat inflammatory glomerular injury can be demonstrated in a rat nephrotoxic serum nephritis model according to the method of Kawasaki, *et al.*, *J. Immunol.*, 150:1074-1083 (1993).

15 The ability of the negative regulators to treat radiation-induced enteritis can be demonstrated in a rat abdominal irradiation model according to the method of Panes, *et al.*, *Gastroenterology*, 108:1761-1769 (1995).

20 The ability of the negative regulators to treat radiation pneumonitis can be demonstrated in a murine pulmonary irradiation model according to the method of Hallahan, *et al.*, *Proc. Natl. Acad. Sci (USA)*, 94:6432-6437 (1997).

The ability of the negative regulators to treat reperfusion injury can be demonstrated in the isolated heart according to the method of Tamiya, *et al.*, *Immunopharmacology*, 29:53-63 (1995), or in the anesthetized dog according to the model of Hartman, *et al.*, *Cardiovasc. Res.* 30:47-54 (1995).

25 The ability of the negative regulators to treat pulmonary reperfusion injury can be demonstrated in a rat lung allograft reperfusion injury model according to the method of DeMeester, *et al.*, *Transplantation*, 62:1477-1485 (1996), or in a rabbit pulmonary edema model according to the method of Horgan, *et al.*, *Am. J. Physiol.* 261:H1578-H1584 (1991).

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The ability of the negative regulators to treat stroke can be demonstrated in a rabbit cerebral embolism stroke model according to the method of Bowes, *et al.*, *Exp. Neurol.*, 119:215-219 (1993), in a rat middle cerebral artery ischemia-reperfusion model according to the method of Chopp, *et al.*, *Stroke*, 25:869-875 (1994), or in a rabbit reversible spinal cord ischemia model according to the method of Clark *et al.*, *Neurosurg.*, 75:623-627 (1991). The ability of the negative regulators to treat cerebral vasospasm can be demonstrated in a rat experimental vasospasm model according to the method of Oshiro, *et al.*, *Stroke*, 28:2031-2038 (1997).

The ability of the negative regulators to treat peripheral artery occlusion can be demonstrated in a rat skeletal muscle ischemia/reperfusion model according to the method of Gute, *et al.*, *Mol. Cell Biochem.*, 179:169-187 (1998).

The ability of the negative regulators to treat graft rejection can be demonstrated in a murine cardiac allograft rejection model according to the method of Isobe, *et al.*, *Science*, 255:1125-1127 (1992), in a murine thyroid gland kidney capsule model according to the method of Talento, *et al.*, *Transplantation*, 55:418-422 (1993), in a cynomolgus monkey renal allograft model according to the method of Cosimi, *et al.*, *J. Immunol.*, 144:4604-4612 (1990), in a rat nerve allograft model according to the method of Nakao, *et al.*, *Muscle Nerve*, 18:93-102 (1995), in a murine skin allograft model according to the method of Gorczynski and Wojcik, *J. Immunol.* 152:2011-2019, (1994), in a murine corneal allograft model according to the method of He, *et al.*, *Ophthalmol. Vis. Sci.*, 35:3218-3225 (1994), or in a xenogeneic pancreatic islet cell transplantation model according to the method of Zeng, *et al.*, *Transplantation*, 58:681-689 (1994).

The ability of the negative regulators to treat graft-vs.-host disease (GVHD) can be demonstrated in a murine lethal GVHD model according to the method of Harning, *et al.*, *Transplantation*, 52:842-845 (1991).

The ability of the negative regulators to treat cancers can be demonstrated in a human lymphoma metastasis model (in mice) according to the method of Aoudjit, *et al.*, *J. Immunol.*, 161:2333-2338, (1998).

Regulatory Binding Site

The invention also provides an LFA-1 regulatory binding site. The regulatory binding site is displayed on the α_L chain of LFA-1 in its wild type, or native, conformation. Fragments of the α_L chain that display the regulatory site are also contemplated, and preferred fragments of the invention include α_L chain I domain sequences, as well as fragments consisting of less than a complete α_L chain I domain. The invention provides LFA-1 regulatory binding sites as part of a polypeptide comprising a human LFA-1 amino acid sequence, the amino acid sequence of a species homolog of human LFA-1, the amino acid sequence of analogs of human LFA-1, or the amino acid sequence of a synthetic polypeptide with homology to human LFA-1. Regulatory binding sites displayed on synthetic polypeptide-like mimetics are also contemplated.

The regulatory binding site of the invention binds a diaryl sulfide (alternatively referred to as a diaryl thioether compound) comprising a first aryl ring and second aryl ring linked to one another through a sulfur atom. In one aspect, the site is defined by human LFA-1 amino acid residues Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹ and Ile³⁰⁶. Alternatively, the binding site is defined by other amino acid residues (*i.e.*, conservative substitutions) or compounds that mimic the binding ability of a site defined by LFA-1 Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹ and Ile³⁰⁶. The regulatory site is also defined by LFA-1 α_L polypeptide amino acid residues that present a domain that binds (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)-phenyl]sulfide. Preferably, the regulatory site of the invention reversibly binds a negative regulator compound.

The invention also provides LFA-1 regulatory binding site mutants wherein one or more amino acid residues defining the site (*i.e.*, presenting the (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]-sulfide binding site) is substituted with an alternative amino acid residue, wherein substitution of the wild type amino acid residues results in modified capacity for the mutant to bind (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)-ethenyl)phenyl]sulfide compared to a wild type regulatory site. Preferred mutant regulatory sites exhibit modified affinity and/or avidity for ICAM-1, both in the

presence and absence of an agent that induces ICAM-1 binding (*e.g.*, the monoclonal antibody 240Q which induces LFA-1 into an activated state required for ICAM binding). Presently preferred mutants include (i) those demonstrating wild type levels of ICAM-1 binding with or without monoclonal antibody 240Q induction, exemplified by mutants having one or more of the amino acid changes Val¹⁵⁷-Ala, Glu²¹⁸-Ala, Thr²³¹-Ala, Lys²⁸⁰-Ala, and Lys²⁹⁴-Ala, (ii) mutants that support greater than wild type levels of binding without induction and wild type levels with induction, exemplified by mutants having one or more of the amino acid changes Ile²³⁵-Ala, Ile²⁵⁵-Ala, Ser²⁸³-Ala, Glu²⁸⁴-Ala, Glu³⁰¹-Ala, and Ile³⁰⁶-Ala, (iii) mutants with decreased levels of ICAM-1 binding relative to wild type in the absence of induction, but wild-type levels with antibody 240Q induction, exemplified by mutants having one or more of the amino acid substitutions Lys¹⁶⁰-Ala, Lys²³²-Ala, Asp²⁵³-Ala, Lys²⁸⁷-Ala, Gln³⁰³-Ala, Lys³⁰⁴-Ala, and Lys³⁰⁵-Ala, and (iv) mutants demonstrating severely decreased levels or no ICAM-1 binding with or without induction, exemplified by a mutant with the substitution Tyr³⁰⁷-Ala.

Mutants of the LFA-1 regulatory site are useful in production of antibodies that more precisely define LFA-1 epitopes that can serve as targets for therapeutic intervention. As another example, soluble regulatory sites (or LFA-1 regulatory sites as part of chimeric compounds) with an increased ability to bind an ICAM that binds LFA-1 can modulate LFA-1 binding to the ICAM through competitive inhibition.

Screening Methods

The invention further provides methods for identifying a negative regulator of LFA-1 binding to an ICAM that binds LFA-1 comprising the steps of (i) contacting LFA-1 and the ICAM in the presence and absence of a test compound under conditions that allow binding of LFA-1 to the ICAM, (ii) identifying as a negative regulator the compound which decreases LFA-1 binding to the ICAM and which binds LFA-1 α_1 polypeptide at a site presenting a diaryl sulfide binding conformation defined by Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹, and Ile³⁰⁶ of human LFA-1. An IC₅₀ value for a compound is defined as the concentration of the compound

required to produce 50% inhibition of a biological activity of interest. As used herein, a negative regulator is defined as a compound characterized by an IC_{50} for inhibition of LFA-1 binding to a natural ligand. Negative regulators of LFA-1 binding are defined to have an IC_{50} of less than about 200 μM , less than about 100 μM , less than about 50 μM , and preferably from about 0.05 μM to 40 μM . In another aspect, the invention provides methods for identifying a negative regulator of LFA-1 binding to an ICAM that binds LFA-1 comprising the steps of (i) contacting LFA-1 and the ICAM under conditions that allow binding of LFA-1 to the ICAM in the presence and absence of a test compound, (ii) identifying as a negative regulator the compound which decreases LFA-1 binding to the ICAM and which competes with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide for binding to LFA-1 α_L polypeptide. Alternatively, the negative regulator competes with 4-amino-2-chlorophenyl-(1'-chloro-2-naphthylphenyl)-sulfide for binding to LFA-1 α_L polypeptide.

In addition, the regulatory site is defined as the site binding site for a negative regulatory that competes for binding to LFA-1 with one of 3-chloro-4-(1-chloro-naphthalen-2-ylsulfanyl)-phenylamine, 2-iso-propylphenyl[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide, (4-methylphenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide, 3-chloro-4-(2-chloro-4-(N,N-dimethylamino)-phenylsulfanyl)-phenylamine, [3-chloro-4-(4-isopropylphenyl-sulfanyl)phenyl]methylaniline, (2,4,-dichlorophenyl)[2-chloro-4-(E-((3-(1-pyrrolidin-2-onyl)propylamino)carbonyl)ethenyl)phenyl]sulfide, (2-methylphenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide, (2-formylphenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide, and 1-[4-(2-isopropyl-phenylsulfanyl)-piperidin-1-yl]ethanone.

The invention also provides methods to identify candidate compounds particularly useful as negative regulators of LFA-1 binding to an ICAM that binds LFA-1 comprising the steps of (i) contacting LFA-1 with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide in the presence and absence of a compound, and (ii) identifying the compound as a putative negative regulator wherein the compound competes with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-

acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide for binding to the LFA-1 α_L polypeptide. The invention therefore provides a method to screen for candidate negative regulators and/or to confirm the mode of action of compounds that decrease LFA-1 binding to an ICAM.

5 The methods of the invention to identify negative regulators are particularly amenable to the various high throughput screening techniques known in the art. There are a number of different libraries used for the identification of small molecule modulators in these screening techniques of the invention, including, (1) chemical libraries, (2) natural product libraries, and (3) combinatorial libraries
10 comprised of random peptides, oligonucleotides or organic molecules. Chemical libraries consist of structural analogs of known compounds or compounds that are identified as "hits" or "leads" via natural product screening. Natural product libraries are collections of microorganisms, animals, plants, or marine organisms which are used to create mixtures for screening by: (1) fermentation and extraction of broths
15 from soil, plant or marine microorganisms or (2) extraction of plants or marine organisms. Natural product libraries include polyketides, non-ribosomal peptides, and variants (non-naturally occurring) thereof. For a review, see *Science* 282:63-68 (1998). Combinatorial libraries are composed of large numbers of peptides, oligonucleotides or organic compounds as a mixture. They are relatively easy to
20 prepare by traditional automated synthesis methods, PCR, cloning or proprietary synthetic methods. Of particular interest are peptide and oligonucleotide combinatorial libraries. Still other libraries of interest include peptide, protein, peptidomimetic, multiparallel synthetic collection, recombinatorial, and polypeptide libraries. For a review of combinatorial chemistry and libraries created therefrom, see
25 Myers, *Curr. Opin. Biotechnol.* 8:701-707 (1997). Identification of modulators through use of the various libraries described herein permits modification of the candidate "hit" (or "lead") to optimize the capacity of the "hit" to modulate activity.

 In high throughput screening methods embraced by the invention, robotic methods are contemplated wherein libraries comprising tens to hundreds of
30 thousands of compounds can be rapidly and efficiently screened.

The invention further provides novel compounds identified as negative regulators in methods of the invention. Negative regulators of the invention are compounds that decrease LFA-1 binding to an ICAM (as compared to binding in the absence of the compound) and compete with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide for binding to the α_1 polypeptide of LFA-1. Presently preferred inhibitors are substituted diaryl sulfides. Exemplary compounds include those as described in co-pending U.S. patent applications entitled "Cell Adhesion-Inhibiting Antiinflammatory and Immune Suppressive Compounds" filed April 2, 1999, attorney docket number 6446.US.Z3, Serial Number 09/286,645, incorporated herein by reference in its entirety, and "Inhibitors of LFA-1 Binding to ICAMs and Uses Thereof" filed April 2, 1999, attorney docket number 27866/35374, USSN 09/285,325, incorporated herein by reference in its entirety.

The invention also provides compositions comprising negative regulators of the invention, and preferably pharmaceutical compositions further comprising a pharmaceutically acceptable diluent or carrier. Pharmaceutical compositions are particularly useful for treatment of a variety of pathological disorders in humans or other animals, *e.g.*, disorders amenable to animal models as described above.

The invention further provides use of a negative regulator identified by a method of the invention in the production of a medicament to ameliorate pathologies arising from LFA-1 binding to an ICAM that binds LFA-1.

The invention also provides kits to identify inhibitors of LFA-1 binding to an ICAM that binds LFA-1, comprising one or more of a purified and isolated LFA-1 polypeptide, a purified and isolated ICAM polypeptide that binds LFA-1, cells expressing LFA-1, and cells expressing the ICAM. Appropriate control reagents and buffers are contemplated in kits of the invention.

The present invention is illustrated by the following examples.

Example 1 describes a high throughput assay to identify inhibitors of LFA-1 binding to full length ICAM-1. Example 2 relates to identification of LFA-1 residues that

participate in antagonist binding. Example 3 describes production of an antibody that activates LFA-1. Example 4 describes identification of an ICAM-1 binding site on LFA-1.

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Example 1 **High Throughput Screening for LFA-1/ICAM-1 Binding Inhibitors**

In an effort to identify inhibitors of LFA-1/ICAM-1 binding, a high throughput screening (HTS) assay was designed to efficiently screen large numbers of chemical compounds in a proprietary library as follows.

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Preliminary experiments were carried out in order to define the linear range of LFA-1/ICAM-1 interaction. Recombinant ICAM-1/IgG1 fusion protein (comprising full length ICAM-1) was prepared as described in U.S. Patent Nos. 5,770,686, 5,837,478, and 5,869,262, each of which is incorporated herein by reference. The extracellular domain of ICAM-1 was subcloned into plasmid pDC1 by standard methods to generate an expression construct encoding a chimeric protein containing the ICAM-1 extracellular domain fused to the Fc region of the heavy chain of human IgG1 just upstream of the hinge. The protein was expressed in CHO cells and purified using protein A Sepharose[®]. The fusion protein was biotinylated using a kit obtained from Pierce Chemical (Rockford, IL). Biotinylated protein (BioIgICAM-1) concentration was determined by measuring absorbance at 280 nm, and serial dilutions were prepared to give a final concentration range of 50 µg/ml to 0.008 µg/ml. Titration of BioIgICAM-1 was carried out with the protein first aliquoted into wells on an assay plate. Recombinant LFA-1 was added to each well at the same concentration and the experiment (as described below) was carried out to completion. The amount of binding was determined for each well, and from a subsequent plot of the results, a single concentration of BioIgICAM-1 was selected for subsequent experiments. In a similar manner, LFA-1 was titrated using the BioIgICAM-1 concentration selected as described above.

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On day 1 of the HTS procedure, the capture antibody, *i.e.*, a non-blocking anti-LFA-1 monoclonal antibody (TS2/4.1; ATCC #HB244), was diluted in plate coating buffer (50 mM sodium carbonate/bicarbonate, 0.05% ProClin[®] 300, pH 9.6) to a final concentration of 2 µg/ml. Immulon[®] 4 (Dynex

Technologies. Chantilly, VA) plate wells were coated with 100 μ l diluted antibody solution per well, and incubation was carried out overnight at 4°C. On day 2, the plates were warmed to room temperature and washed two times with wash buffer (calcium- and magnesium-free phosphate buffered saline, CMF-PBS) with 0.05% Tween[®]-20). To each well, 200 μ l of blocking solution (5% fish skin gelatin in CMF-PBS with 0.05% ProClin[®] 300) was added, and the blocking incubation was carried out at room temperature for 30 min. The blocking solution was removed by aspiration, and the plates were not washed. LFA-1 was diluted to a final concentration of 1 μ g/ml in assay buffer (1% fish skin gelatin and 2 mM MgCl₂ in CMF-PBS), and 100 μ l was added to each well. Incubation was carried out for one hour, and the plates were washed two times with wash buffer.

A 2X stock solution of BioIgICAM-1 was prepared containing 0.1 μ g/ml BioIgICAM-1 and 4 μ M crystal violet (an activator of LFA-1/ICAM-1 binding) in Assay Buffer (EG&G Wallac, Gaithersburg, MD). Diluted aliquots (50 μ l) of pooled chemicals (22 compounds/pool) from the chemical library were added to the wells, followed by addition of 50 μ l of the 2X stock of BioIgICAM-1 to provide a final assay volume of 100 μ l (containing 2% DMSO). The plates were incubated for one hour at room temperature and washed once with wash buffer. Europium-labeled streptavidin (Eu-SA; #1244-360, EG&G Wallac) was diluted 1:500 in Assay Buffer, 100 μ l of the diluted Eu-SA was added to each well, and the plates were incubated at room temperature for one hour.

Plates were washed eight times with wash buffer, 100 μ l of DELFIA[®] enhancement solution (EG&G Wallac) diluted 1:2, was added to each well, and the plates were shaken for five minutes using a Wallac shaker at fast speed. Plates were read using a Wallac DELFIA[®] fluorescence reader (fluorimeter). Controls included both positive and negative wells and 50% binding wells established using blocking antibodies, *i.e.*, anti-LFA-1 monoclonal antibody (TS1/22.1, ATCC #HB202) or an anti-ICAM-1 monoclonal antibody. Chemical pools in wells showing 50% or greater inhibition of LFA-1 binding to ICAM-1 were identified and the experiment was repeated using individual chemicals from those pools. Inhibitors of LFA-1/ICAM-1 binding were identified, and a further screen was performed to determine dose

dependence of the inhibitory activity. Further study of selected compounds was carried out using biochemical and cellular assay techniques.

The HTS assay identified more than 40 compounds as hits demonstrating potency in inhibiting LFA-1/ICAM-1 interaction. Of these many, compounds exhibited a diaryl sulfide structure, thereby identifying these compounds as a class of LFA-1/ICAM-1 binding inhibitors.

Example 2

Identification of an LFA-1 Regulatory Binding Site

A. LFA-1 Antagonist Binding to the LFA-1 I domain

Nuclear magnetic resonance (NMR) spectroscopy has proven to be a useful technique to detect small molecule binding to proteins. This technique for screening, or establishing the structure activity relationship (SAR) by NMR [Shuker, *et al.*, *Science* 274:1531-1534 (1996), incorporated herein by reference], has been successful to identify drug leads against several proteins [WO 97/18471, published May 22, 1997 and WO 97/18469, published May 22, 1997, both of which are incorporated herein by reference]. This technique relies on detecting chemical shifts of amide proton and nitrogen atoms resulting from changes in the chemical environment of the peptide backbone, such as those that occur upon ligand binding. Based on the technique's sensitivity, experiments were designed to evaluate binding of small molecule antagonists to LFA-1 in order to understand the structural basis for chemical inhibition of LFA-1 binding to ICAM-1.

Intact LFA-1 is too large to study by NMR spectroscopy. However, evidence indicates that the α_1 chain I domain of LFA-1 is largely responsible for ICAM-1 binding, and recombinant I domain polypeptides can compete with intact LFA-1 for ICAM-1 binding. The approximately 200 amino acid I domain region was therefore subcloned, and the recombinant polypeptide was used in NMR experiments to assess whether antagonists of LFA-mediated adhesion interact with the I domain.

The I domain polypeptide corresponding to residues 127-309 in SEQ ID NO: 1 was isotopically labeled in *E. coli* and purified. The pET15b plasmids encoding residues 127-310, 127-309, or 127-305 of SEQ ID NO: 2 were prepared by

PCR amplification of the respective sequences using the human LFA-1 gene as a template and cloned using standard techniques. Each expression plasmid was checked by sequencing.

For NMR experiments, uniformly ^{15}N - or ^{15}N -, ^{13}C -labeled protein was prepared by growing the *E. coli* strain BL21 (DE3) overexpressing the I domain of LFA-1 on M9 medium containing $^{15}\text{NH}_4\text{Cl}$ with or without $[\text{U-}^{13}\text{C}]$ -glucose. In addition, $[\text{U-}^{15}\text{N}, \text{H}]$ -labeled proteins, with $[\text{U-}^{13}\text{C}]$ -labeled methyl protons in valine and leucine, were prepared to facilitate the interpretation of ^{13}C NOESY experiments [Gardner and Kay, *J. Am. Chem. Sci.* 119:7599 (1997)]. The recombinant I domain was purified using nickel affinity resin according to the manufacturer's suggested protocol. The NMR samples contained 0.8 mM protein, 100 mM sodium phosphate, pH 7.2, in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1) or 99.9% D_2O .

All NMR spectra were acquired at 30°C on Bruker DRX500 or DRX600 NMR spectrometers. Backbone resonances were assigned using the HNCA, HN(CO)CA, HN(CA)CB, HN(COCA)CB, HNCO and HN(CA)CO triple resonance experiments using uniformly ^{15}N , ^{13}C labeled protein. Sidechain assignments were made using the HACACO, HBHA(CO)NH, ^{15}N Edited TOCSY and the HCCH-TOCSY three dimensional experiments. Distance restraints were obtained from ^{13}C -resolved 3D NOESY and ^{13}C edited-filtered NOESY experiments.

Models for the bound compounds were generated with a simulated annealing protocol using the program XPLOR. The docking calculations were performed using the NMR derived distance constraints. The starting protein coordinates in these calculations were derived from the x-ray crystal structure [Qu and Leahy, *Proc. Natl. Acad. Sci.(USA)* 92:10277-10281 (1995)]. Starting structures for the compound were generated randomly. The backbone atoms of the protein were fixed in the docking calculations.

The two dimensional heteronuclear single quantum correlation (HSQC) spectra of the ^{15}N -labeled I domain was indicative of a folded structure. Addition of (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide induced multiple chemical shift changes in the

LFA-1 domain spectrum thereby confirming that the I domain of LFA-1 binds to this antagonist.

B. Binding Interface of Small Molecule Ligand with LFA-1 I domain

5 To identify the amino acids whose chemical shifts were perturbed by the antagonist, *i.e.*, to map the negative regulator binding site, backbone and side chain resonance assignments of the protein were made using standard heteronuclear NMR experiments. The secondary structure of truncated LFA-1 I-domain protein was compared to that of the x-ray crystal structure of the I domain in intact LFA-1 [Qu and Leahy, *supra*], using both nuclear Overhauser effects (NOE) and backbone chemical shifts. Data indicated that the secondary structure of the I domain in the truncated protein was identical to that found in the previously defined LFA-1 crystal structure. As a result, the antagonist-induced chemical shift changes, as determined by NMR, could then be reliably mapped onto the three-dimensional structure of the I domain, as determined by x-ray crystallography.

15 In these studies, the largest chemical shift changes occurred for residues that lined a cleft between the carboxy terminal helix of the I domain and central beta sheets. Residues adjacent the metal binding site (MIDAS) showed no shift upon negative regulator binding.

20 More detailed analysis of (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetyl)piperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide binding to LFA-1 was based on NOE experiments. Protons of the protein and negative regulator that were within 5 Å of each other were identified. Resonances that shifted upon negative regulator binding were reassigned by following the shift changes that occurred during a titration of ligand binding and by comparing the pattern of NOEs observed between protons on the protein in the presence and absence of negative regulator. Both ¹³C edited and ¹³C edited-filtered NOE experiments were used to identify NOEs between the negative regulator and protein.

25 Thirty-nine regulatory site contacts were identified and used to dock the negative regulator into the protein. The inter-protein NOEs that were observed in the complex are similar to those predicted by the crystal structure for the I domain.

Based on this observation, the negative regulator/free crystal structure coordinates were used as the starting conformation for the protein in a proposed model of the protein/regulator complex.

Negative regulators were docked using the NOE constraints and the program XPLOR. In the docking calculations, the protein backbone was kept rigid, but amino acid sidechains of the protein were allowed to relax to accommodate the ligand. Only minor changes in protein conformation were necessary to dock the regulator. In all of the docking calculations, the negative regulator was found to lie in the cleft between beta sheet 5 and the carboxy terminal helix, alpha 7 in the I domain, in agreement with the model based on chemical shift changes. The top of the binding pocket is formed from the loop connecting alpha helix 7 to beta sheet 5. Negative regulator ring A is positioned to the top part of the cleft by NOE constraints to Ile²⁵⁹ and Leu²⁹⁸ while ring B makes contact to the middle of the cleft with NOEs to Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹ and Ile³⁰⁶. The contacts Val¹⁵⁷ and Leu¹⁶¹ on the helix-5 indicate how deep into the protein pocket ring B sits in the complex. Residue Ile²³⁵ is positioned near the center of the negative regulator and shows large chemical shifts upon regulator binding.

The number of constraints obtained was not sufficient to generate an exhaustively detailed model of the complex. However, the constraints identified unambiguously place the negative regulator binding site in this cleft of the protein. The low number of constraints was due to low sensitivity of NMR signals from residue in the binding pocket that resulted from chemical exchange broadening. Chemical exchange broadening is often indicative of slow motions between different environments. This is generally observed for loose binding of compounds that do not bind in a single conformation. Indeed, some of the constraints observed between the negative regulator and protein were not consistent with a single conformation. For instance, in the docking calculations, two families of possible conformations were found, one pointing toward beta sheet 5 and Ile²⁵⁹, and another with ring A pointing toward alpha helix 7 and residue Leu²⁹⁸.

Results indicated that the protein binding pocket is lined predominantly by hydrophobic Leu/Val/Ile residues. The hydrophobic pocket is,

however, ringed by several hydrophilic groups of lysine and glutamic acid residues. In the ligand-free crystal structure, these hydrophilic groups shield the hydrophobic binding pocket from solvent, possibly by forming salt bridges. In the model for the complex, these hydrophilic side chains move to accommodate the negative regulator.

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Example 3

Production of Activating Monoclonal Antibody 240Q

Female BALB/c mice were immunized with purified recombinant

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α_d /CD18 (described in U.S. Patent 5,837,478, issued November 17, 1998, and incorporated herein by reference). The protein was captured from CHO cell supernatant with a CD18-specific antibody captured on protein A Sepharose[®] beads. Column material (including beads and capture antibody) was injected with incomplete Freund's adjuvant. Four immunizations over a seven month period were performed before animal #2480 was sacrificed for harvest and fusion of the spleen.

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Hybridomas were screened by ELISA for production of IgG by standard protocols. A secondary ELISA screen was performed to identify hybridoma supernatant reactive with either the integrin α or β chain. Briefly, plates were coated in standard buffer with 100 ng/ml of the F(ab')₂ fragment of the CD18-specific antibody, 195N. After a blocking step, CHO cells supernatants containing either soluble α_d /CD18 or CD11a/CD18 were added to the wells and capture of integrins was allowed to continue for four hours at 37°C. Hybridoma supernatants were incubated with bound integrin, after which bound mouse IgG was detected with a horseradish peroxidase-conjugated anti-mouse Fc-specific polyclonal antibody. Hybridomas that reacted with both α_d /CD18 and CD11a/CD18 were presumed to recognize either the common β chain or the leucine zipper region of the recombinant molecule. Supernatants were tested by flow cytometry for recognition of native α_d on α_d -transfected JY cells and HL60 cells. Hybridomas that reacted with neither were presumed to be reactive with the leucine zipper peptides.

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Thirty five hybridomas were identified as CD18-specific in the secondary assay. A tertiary screen was performed to determine whether the antibodies exhibited any function in an adhesion assay measuring the interaction between peripheral blood lymphocytes (PBL) and ICAM-1. Briefly, PBL were isolated from

heparin-treated whole blood by centrifugation on a Ficoll[®] gradient. Monocytes and activated lymphocytes were removed by adherence on plastic. Non-adherent cells were treated with hybridoma supernatants or control antibodies for one hour on ice. As a positive control for activation, phorbol myristate acetate (PMA) was used to stimulate a subset of PBL. Cells were washed once and incubated with ICAM-1 immobilized on microtiter plates. After 45 min at 37°C, bound cells were crosslinked for 12 hr using 2.5% (final concentration) glutaraldehyde. Plates were washed in distilled water and stained with 0.5% (final concentration) crystal violet. Following extensive washing with distilled water, destaining was performed using 66% absolute ethanol. Plates were read on a Beckman ELISA reader with a test filter of 570 nm. Six hybridomas were identified that produced an anti-CD18 monoclonal antibody capable of enhancing PBL binding to ICAM-1 at the same level as the PMA control (three- to four-fold over unstimulated cells). The hybridomas were cloned in successive rounds using a modified limiting dilution method. Five clones survived the cloning process and were retested in the PBL assay and with B and T cells. The antibody 240Q was developed further since it appeared to be more effective at cell stimulation.

Specificity of 240Q was assessed by immunoprecipitation experiments. Biotinylated lysates of HL60 cells, positive for expression of all β_2 integrins, were treated with anti-CD18 antibodies 23I11B, 195N, TS1.18 or with 240Q. Antibody/antigen complexes were isolated with an anti-mouse Ig conjugated to protein A Sepharose[®] matrix. After resolution of protein by SDS-PAGE, biotinylated species were visualized by detection with streptavidin-HRP and developed with a chemiluminescent reagent (Amersham). Antibody 240Q precipitated the identical series of proteins as the known CD18 antibodies. The bands represented known molecular weight proteins for all of the leukointegrin α chains and the CD18 β chain. Extensive immunocytochemical analyses comparing 240Q staining with that of the other anti-CD18 antibodies indicated that 240Q recognized the β chain.

Further evidence that 240Q recognized the β chain (and not a shared epitope on the α chain) was derived from additional immunoprecipitation experiments. It is known that expression of integrins lacking the transmembrane and

cytoplasmic domains results in secretion of large amounts of the free β chain. While the anti-CD18 antibody 195N will bind to and precipitate free β chain, the 23I11B antibody will recognize β chain only in the context of a heterodimer.

Immunoprecipitation of soluble α_v /CD18LZ (leucine zipper) protein from CHO supernatants yields protein that, on SDS-PAGE, is predominantly β chain, with non-stoichiometric amounts of the appropriate alpha chain. In these experiments, the affinity resin is not washed, so disruption of the bound heterodimer would not be expected to affect results.

Several integrin-specific monoclonal antibodies were biotinylated and used in flow cytometry designed to map the 240Q binding site on a coarse level. Cells were incubated with a biotinylated antibody and a different, unlabeled antibody at the same time, and it was determined whether the unlabeled antibody can compete with the labeled antibody. The untreated control consisted of cells stained with the biotinylated antibody alone.

A preliminary experiment was performed to titrate single antibodies with HUT78 (CD11a/CD18⁺ T cell line) and HL60 (CD11a⁺, CD11b⁺, CD11c⁺ myeloid lineage cell line). Biotinylated antibodies were incubated with both cell types at 0.3, 1.0, 3.0, and 10 μ g/ml. Biotinylated antibody was detected with both streptavidin-FITC (to determine whether biotinylation was successful) and anti-mouse Ig/FITC (to determine whether biotinylated antibodies were still functional and binding at equivalent levels). Staining with 240Q with the streptavidin-FITC detection method was only 25% that of the CD18-specific antibody 23I11B at any given concentration. The difference was more dramatic with the anti-mouse FITC detection. Affinity differences would not be expected to account for these results, since transformants stained equally well with both antibodies.

This result implied that 240Q recognizes a specific subset of CD18 molecules on the cell surface, a finding that correlated with previous staining of COS transfectants. Another possibility is that the antibody recognized a particular molecular conformation achieved temporally at only 25% of the time.

In the cross-competition experiments, there appeared to be no overlap between 240Q and any other integrin α or β chain-specific antibodies. This finding

was confirmed by ELISAs with captured recombinant LFA-1 and Mac-1 protein. In these assays, the unlabeled antibody was used to capture the protein and the labeled antibody was used to detect it. Failure of the labeled antibody to bind would indicate that the binding site was occupied by the capture antibody. While 240Q capture
5 blocked binding of biotinylated 240Q, it did not block binding with any other antibody. Capture by CD11a, CD11b, or other CD18 antibodies did not prevent detection by biotinylated 240Q. There was no difference between the ability of 240Q and other CD18-specific antibodies to recognize recombinant CD18 integrins. Treatment of immobilized recombinant LFA-1 with either 240Q or manganese did not
10 enhance ICAM-1 binding, implying that the recombinant integrin was in a constitutively activated conformation.

Based on the observation that 240Q treatment of cells in the cross-blocking experiments caused aggregation, an aggregation assay was run with JY, Jurkat, and HL60 cells. Cells were plated in culture medium and treated with
15 concentrations of 240Q or 195N ranging from 0.2 to 10 µg/ml. After a 30 minute incubation at 37°C, wells were photographed. Antibody 240Q at concentrations from 0.5 - 10 µg/ml appeared to induce substantial aggregation. Antibody 195N did not induce the aggregate phenotype. It was not apparent whether this behavior was due to integrin-CAM interactions or an indirect induction of other adhesive pathways.

The integrin-activation function of 240Q was further characterized in binding experiments using the TACO cell line. These cells were isolated from a patient diagnosed with a subtype of leukocyte adhesion deficiency (LAD). The subtype is characterized by normal surface expression of LFA-1 on lymphocytes, but the inability of LFA-1 to bind ICAM-1. The functional phenotype is not recognized
20 by phorbol myristate acetate (PMA). Treatment of the cells with the antibody 240Q rescued homotypic aggregation, which was determined to be ICAM-1-dependent using an ICAM-1-specific antibody. When subsaturating amounts of the antibody F(ab')₂ fragment were used to treat the cells, aggregation did not occur and the 240Q-treated cells were capable of recognizing ICAM-1/Fc protein immobilized on
25 microtiter plates. Cells which were treated with the anti-CD18 antibody and either no
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and the protein was concentrated ten-fold. Concentration of the protein was determined by absorption at 280 nm using an extinction coefficient of 1.0 AU/1.4 mg of ICAM-1 domain 1 and 2. The purification process and analysis was repeated using the same column and an additional 2.5 liters of CHO culture supernatant. The two
5 pools were combined and filtered.

B. ICAM-1 Binding Interface on the LFA-1 I-Domain

Residues that are important for ICAM-1 binding to the LFA-1 I domain have previously been identified using mutational studies and residues that
10 form the MIDAS region of the I domain have been shown to be important for binding by this approach. Other LFA-1 regions have been investigated using chimeric proteins comprising human and mouse I domains [Huang and Springer, *J. Biol. Chem.* 270:19008-19016 (1995)]. Because many of the residues in the LFA-1 ligand binding site important for ICAM-1 binding are either identical (Tyr³⁰⁷, Lys³⁰¹, Lys²⁸⁷) or highly
15 conserved (human Lys³⁰⁵, Lys³⁰⁴ - mouse Arg³⁰⁵, Arg³⁰⁴) between mouse and human, chimeric protein studies were unable to specifically identify necessary binding residues. Chemical shift changes that occur upon binding provides a sensitive way to map binding sites. ¹H-¹³C HSQC and ¹H-¹⁵N HSQC spectra of the I domains of
LFA-1 in the presence and absence of ICAM-1 were used to identify residues affected
20 by ICAM-1 domains 1 and 2 fragment binding using NMR techniques as generally described above.

The complex was found to be in slow exchange on the NMR timescale, indicating binding much tighter than 10 μ M. Many residues whose NMR signals show the largest changes upon binding were found on the surface of the I
25 domain. In addition, residues near the MIDAS motif and alpha helix 7 of the small molecule ligand binding site were most affected by ICAM-1 binding. These data indicate that the MIDAS motif and alpha helix 7 participate in ICAM-1 binding, either directly by binding the ligand, or indirectly by mediating a conformational change in the I domain. Furthermore, the involvement of the α helix 7 in ICAM-1
30 binding provides a rationale for how small molecules that bind to this region of the I domain disrupt LFA-mediated adhesion.

C. Functionally Important Residues in the Ligand Binding Pocket

In an attempt to identify functional I domain residues in and around the site of compound binding, amino acid substitution mutants were generated and tested for the ability to bind ICAM-1. Amino acids most affected in NMR by compound binding and whose side chains are directed toward the surface of the I domain were substituted with alanine. In addition, Asp¹³⁷, a residue located within and essential to a functional MIDAS and ICAM-1 binding site, was substituted with alanine. The various I domain mutants were expressed in COS cells and cell adhesion to ICAM-1 was determined in the presence of a CD18 monoclonal antibody, 240Q, that induces high avidity binding.

1. Generation of the mutations in the CD11a I domain:

Twenty-five individual mutations in the LFA-1 α polypeptide (CD11a) were generated. Each mutation was prepared using Stratagene's QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Briefly, two primers were synthesized that introduced a specific mutation in the amplification product. Primers utilized are set out below, with only the sense primer shown.

D137A/S: SEQ ID NO: 5

CTGGTATTTCTGTTTGCGGGTTCGATGAGCTTG

V157A/S: SEQ ID NO: 6

GACTTCATGAAGGATGCGATGAAGAACTCAGC

K160A/S: SEQ ID NO: 7

GAAGGATGTGATGAAGGCGCTCAGCAACACTTGC

E218A/S: SEQ ID NO: 8

CAATTATGTCGCGACAGCGGTGTTCCGGGAGGAG

T231A/S: SEQ ID NO: 9

GCCCCGCCAGATGCCGCGAAAGTGCTTATCATC

K232A/S: SEQ ID NO: 10

CGGCCAGATGCCACCGCGGTGCTTATCATCATC

I235A/S: SEQ ID NO: 11
GCCACCAAAGTGCTTGCATCATCACGGATGGG

D253A/S: SEQ ID NO: 12
CATCGATGCGGCCAAAGCGATCATCCGCTACATC

5 I255A/S: SEQ ID NO: 13
GCGGCCAAAGACATCGCGCGCTACATCATCGGG

K280A/S: SEQ ID NO: 14
CACAAATTTGCATCAGCGCCCGCGAGCGAGTTTG

S283A/S: SEQ ID NO: 15
10 GCATCAAAACCCGCGGCGGAGTTTGTGAAAATTC

E284A/S: SEQ ID NO: 16
CAAAACCCGCGAGCGCGTTTGTGAAAATTCTG

K287A/S: SEQ ID NO: 17
GCGAGCGAGTTTGTGGCGATTCTGGACACATTG

15 K294A/S: SEQ ID NO: 18
CTGGACACATTTGAGGCGCTGAAAGATCTATTC

E301A/S: SEQ ID NO: 19
GAAAGATCTATTTACTGCGCTGCAGAAGAAGATC

Q303A/S: SEQ ID NO: 20
20 CTATTTACTGAGCTGGCGAAGAAGATCTATGTC

K304A/S: SEQ ID NO: 21
TTCACTGAGCTGCAGGCGAAGATCTATGTCATTG

K305A/S: SEQ ID NO: 22
CACTGAGCTGCAGAAGGCGATCTATGTCATTGAG

25 I306A/S: SEQ ID NO: 23
GAGCTGCAGAAGAAGGCGTATGTCATTGAGGGC

Y307A/S: SEQ ID NO: 24
CTGCAGAAGAAGATCGCGGTCATTGAGGGCACA

30 Control mutants included the following, wherein amino acid changes were introduced in regions reported by others to be involved in ICAM-1 binding.

T243A/S: SEQ ID NO: 25

ACGGATGGGGAGGCCGCGGACAGTGGCAACATC

S245A/S: SEQ ID NO: 26

GGGGAGGCCACTGACGCGGGAAACATCGATGC

5 N247A/S: SEQ ID NO: 27

GCCACTGACAGTGGCGCGATCGATGCGGCCAAAG

D249A/S: SEQ ID NO: 28

GACAGTGGCAACATCGCGGCGGCCAAAGACATC

K252A/S: SEQ ID NO: 29

10 CAACATCGATGCGGCCGCGGACATCATCCGCTAC

The primers were used in two PCR reactions, one with full-length CD11a (residues 1-1170) in plasmid pDC1 as template and the other with CD11a I domain (residues 152-334) in plasmid pET15b as template. PCR reaction conditions varied depending on the melting temperature (T_M) of the primers. Details of the reaction for each mutation are described below. The general format was as follows: one cycle at 95°C for 30 seconds followed by 16 cycles of: 95°C for 30 seconds, 55°C for one minute, and 68°C for 20 minutes. After PCR, template DNA was digested with *DpnI* at 37°C for one hour and the remaining amplified DNA was used to transform supercompetent *E. coli* XL1 Blue (Stratagene) according to the manufacturer's suggested protocol. Selected colonies were grown in liquid culture and plasmid DNA was isolated and sequenced. For the full-length mutants, a 1.8 kb *HindIII/EcoRI* fragment containing the 5' half of the gene was isolated and subcloned into the parental vector. Subclones were sequenced to verify the integrity of the junctions and the presence of the mutation.

2. PCR Conditions

Mutations V157A, E218A, T231A, I235A, I255A, E284A, K287A, K294A, K305A were generated in PCR including a 45°C annealing step and a 58°C extension step. In generating these mutations, extension times for the full length sequence in pDC1 was 20 minutes and 15 minutes for I domain in pET15b.

For mutations D137A, K160A, K232A, K280A, S283A, E301A, Q303A, K304A and I306A, the same temperatures as described above were used, but with both templates, the extension time for both templates was 20 minutes. For mutants Y307A and D253A, an extension step of 25 minutes was used.

For mutants T243A, S245A, N247A, D249A and K252A, the annealing step was carried out at 45°C, and extension was carried out at 60°C for 20 minutes. For mutant S245A, PCR included 18 cycles rather than 16 cycles.

3. COS Cell Transfections

On day 1, COS cells were seeded at 1.6×10^6 cells per 10 cm plate in DMEM 10% FBS (growth media). After 18 to 24 hr, cells were transfected as follows. Seven µg each of CD18/pDC1 and CD11a/pDC1 plasmid DNA was added to three ml OPTI MEM media and 49 µl Lipofectamine was added to another three ml of the same media. The two resulting solutions were mixed, inverted five times, incubated at room temperature for 30 min, and diluted by addition of 6.1 ml OPTI MEM. Cells were washed once with OPTI MEM and the DNA/Lipofectamine mixture was added. Cells in the mixture were incubated at 37°C in CO₂ for six hours. Media containing the plasmid DNA was removed and replaced with growth media. Cells were grown overnight and media was removed and replaced. After overnight growth, cells were split 1:2 and grown overnight again. Cells were removed from the plate with Versene, collected by centrifugation, resuspended in adhesion buffer containing (RPMI containing 5% inactivated FBS), and counted. Cells were then used for adhesion assays and for fluorescence activated cell sorting (FACS) staining and analysis.

4. Adhesion Assay

Adhesion assays were performed in 96-well Easy Wash plates (Corning, Corning NY) using a modification of a previously reported procedure [Sadhu, *et al.*, *Cell. Adhes. Commun.* 2:429-440 (1994)]. Each well was coated overnight at 4°C with (i) 50 µl of ICAM-1/Fc (5 µg/ml), (ii) anti-CD18 monoclonal antibody TS1/18 [Sanchez-Madrid, *et al.*, *Proc. Natl. Acad. Sci. (USA)* 79:7489-7493 (1982); Weber, *et al.*, *J. Immunol.* 159:3968-3975 (1997); Lu, *et al.*, *J. Immunol.*

159:268-278 (1997)] at 5 µg/ml together with anti-CD11a monoclonal antibody TS1/22 at 5 µg/ml in 50 mM bicarbonate buffer, pH 9.6, or (iii) buffer alone. Plates were washed twice with 200 µl per well D-PBS and blocked with 1% BSA (100 µl/well) in D-PBS for one hour at room temperature. Wells were rinsed once with 100 µl adhesion buffer (described above) and 100 µl adhesion buffer was then added to each well. Adhesion buffer (100 µl) with or without blocking antibody TS1/22 at 20 µg/ml was added to each well. COS transfectants (100 µl, approximately 0.75 x 10⁶ cells/ml) expressing the heterodimer (with or without a mutation) in adhesion buffer, with or without activating antibody 240Q (10 µg/ml) was added to each well and the plates incubated at 37°C for 15 to 20 min. Adherent cells were fixed by addition of 50 µl/well 14% glutaraldehyde in D-PBS and incubated at room temperature for 1.5 hr. The plates were washed with dH₂O and stained with 100 µl/well 0.5% crystal violet in 10% ethanol for five minutes at room temperature. Plates were washed in several changes of dH₂O. After washing, 70% ethanol was added, and adherent cells were quantitated by determining absorbance at 570 nm and 410 nm using a SPECTRAMax[®] 250 microplate spectrophotometer system (Molecular Devices, Sunnyvale, CA). Percentage of cells binding was calculated using the formula:

$$\% \text{ cells binding} = \frac{A_{570} - A_{410}(\text{binding to ICAM-1})}{A_{570} - A_{410}(\text{binding to CD18 + CD11a antibody})} \times 100$$

Data were normalized using the formula:

$$\% \text{ wildtype binding} = \frac{(\% \text{ mutant cell binding})}{(\% \text{ wildtype cells binding})} \times 100$$

5. FACS Staining

FACs staining was carried out in a 96 well plate. Each transfectant was stained with an antibody to CD18 (TS1/18), an antibody to CD11a (TS1/22), and an activating antibody to CD18 (240Q). Controls included unstained cells, cells

stained with secondary antibody only, and cells stained with an isotype matched control antibody (1B7).

Briefly, approximately 1×10^5 to 5×10^5 cells were aliquoted per well and one antibody per well was added per transfectant. Cells were centrifuged in a
5 table top centrifuge at $1200 \times g$ for five minutes at 4°C , rinsed in staining buffer (containing ice-cold CMF-PBS 2% FBS), and centrifuged again.

Primary antibody (100 μl at 10 $\mu\text{g/ml}$) or staining buffer, was added to each well and incubation carried out on ice for 30 min. Cells were pelleted by centrifugation and washed once with staining buffer. Secondary antibody (100 μl),
10 typically sheep anti-mouse Ig-FITC (Sigma), at a 1:200 dilution was added to each well and incubation carried out on ice in the dark for 30 minutes. Cells were pelleted by centrifugation, washed three times with CMF-PBS, and resuspended and fixed in 300 μl 1% formaldehyde. Samples were analyzed on the same day stained.

Results indicated that the mutants could be separated into four
15 phenotypes: 1) mutants that demonstrated wild type levels of binding with or without 240Q induction (Val¹⁵⁷→Ala, Glu²¹⁸→Ala, Thr²³¹→Ala, Lys²⁸⁰→Ala, and Lys²⁹⁴→Ala), 2) mutants that supported greater than wild type levels of binding without 240Q induction and wild type levels with induction (Ile²³⁵→Ala, Ile²⁵⁵→Ala, Ser²⁸³→Ala, Glu²⁸⁴→Ala, Glu³⁰¹→Ala, and Ile³⁰⁶→Ala), 3) mutants that possessed decreased levels
20 of binding relative to wild type binding in the absence of induction, but wild-type levels with 240Q induction (Lys¹⁶⁰→Ala, Lys²³²→Ala, Asp²⁵³→Ala, Lys²⁸⁷→Ala, Gln³⁰³→Ala, Lys³⁰⁴→Ala, and Lys³⁰⁵→Ala), and 4) mutants that demonstrate severely decreased levels or no binding with or without 240Q (Tyr³⁰⁷→Ala).

The effects of mutations on ICAM-1 binding were not due to varying
25 levels of LFA-1 expression, and both CD11a and CD18 were expressed at equivalent levels to that of wild type. For mutants showing significantly decreased binding, ¹⁵N labeled I domain was prepared and ¹H-¹⁵N HSQC spectra were compared to that of wild type I domain. All of these mutant protein spectra were very similar to that of the wild type protein indicating that no significant conformational changes in the
30 protein arose from any of the mutations. Data for the eighth mutant, K304A, could

not be obtained due to poor expression of the protein in bacteria. These mutants all bound 240Q at equal levels.

The analysis indicates that amino acids in and around the site of antagonist binding contribute to a regulatory site for LFA-1 mediated cell adhesion. The residues Lys²³², Lys²⁸⁷, Lys³⁰⁴, Lys³⁰⁵, and Tyr³⁰⁷ are all hydrophilic residues that surround, but do not directly form, the small molecule ligand binding site. Residues Val¹⁵⁷, Ile²³⁵, Ile²⁵⁵, and Ile³⁰⁶ form the hydrophobic pocket of the small molecule binding site.

6. Mechanism of Regulation

LFA-1 binding activity is regulated through two different mechanisms which are not mutually exclusive: 1) control of individual receptor affinity (the strength of binding between two molecules), and 2) control of overall avidity (the affinity multiplied by the number of interactions which are occurring at one time) by the regulated aggregation of individual receptors through interactions with the cellular cytoskeleton. If the LFA-1 regulatory binding site, as defined above, is responsible for regulating individual receptor affinity, then the activating mutants, typified by I235A (described above), should possess higher binding affinity in cellular adhesion. These methods, however, are imprecise and do not accurately separate affinity from avidity. Therefore, in order to accurately measure the relative binding affinity of wildtype and mutant I235A for ICAM-1, the following assay was carried out. Recombinant I235A was produced in CHO cells in secreted form using the same method as that used for production of recombinant LFA-1 in Example 1. The soluble forms of recombinant LFA-1 (used here and in Example 1) and I235A (used here) contain deletions of the transmembrane and cytoplasmic domains of CD11a and CD18 (SEQ ID NO: 30 [full length polynucleotide] and 31 [full length amino acid], and substitution of these regions for acidic and basic leucine zipper cassettes, respectively, which promote and stabilize specific heterodimerization as described for the production of soluble T-cell receptor [Hsiu-Ching *et al Proc. Natl. Acad. Sci. (USA)* 91: 11408-11412 (1994)].

Both wildtype and mutant I235A CD11a were truncated after position Q1063 in the mature polypeptide, and the 47 amino acid acidic leucine zipper cassette (SEQ ID NO: 32) was added in-frame, using standard methods. CD18 was truncated after position N678 in the mature polypeptide, and the 47 amino acid basic leucine zipper cassette (SEQ ID NO: 33) was added in-frame.

Acidic leucine zipper cassette

SEQ ID NO: 32

Thr Arg Ser Ser Ala Asp Leu Val Pro Arg Gly Ser Thr Thr Ala Pro Ser Ala Gln Leu
Glu Lys Glu Leu Gln Ala Leu Glu Lys Glu Asn Ala Gln Leu Glu Trp Glu Leu Gln
Ala Leu Glu Lys Glu Leu Ala Gln

Basic leucine zipper cassette

SEQ ID NO: 33

Thr Arg Ser Ser Ala Asp Leu Val Pro Arg Gly Ser Thr Thr Ala Pro Ser Ala Gln Leu
Lys Lys Lys Leu Gln Ala Leu Lys Lys Lys Asn Ala Gln Leu Lys Trp Lys Leu Gln Ala
Leu Lys Lys Lys Leu Ala Gln

Both recombinant LFA-1 and I235A were expressed in CHO cells and purified from the supernatants using separate 8 ml immunoaffinity columns created by coupling 2 mg of anti-CD18 23I11B monoclonal antibody per ml of activated CNBr-Sepharose™ according to the manufacturer's suggested protocol, and eluted using a 20 mM Tris (pH 7.5), 2.5 M MgCl₂ buffer. Recombinant LFA-1 and I235A were then purified a second time by gel filtration chromatography over a Pharmacia HiLD SuperDex 200™ column in PBS buffer using standard methods, in order to remove any single chain, aggregated and/or degraded material. The resulting suspensions of purified heterodimers were concentrated using Millipore Ultrafree-4 Centrifugal Filter Units™ with Biomax-30™ membranes, then dialyzed in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, and 2 mM MgCl₂) at 4 °C, and quantitated using a BioRad Protein Assay™ and the manufacturer's protocol.

The affinity of recombinant LFA-1 and I235A was then measured by surface plasmon resonance using a BIAcore 2000 biosensor™ (Pharmacia Biosensor AB). All experiments were performed at 25°C. All proteins for injection were diluted in HBS buffer. Anti-human Fc antibody (Pierce) was coupled to a CM5™ sensor chip (Pharmacia Biosensor AB) using an amine-coupling kit (Pharmacia

Biosensor AB). The antibody was injected at 50 mg/ml in 10 mM Na acetate (pH 4.5) buffer until approximately 12,000 RU was bound. For each assay, recombinant ICAM-1/IgG1 (see above) was injected at 10 mg/ml until 200 RU was captured onto the chip through binding to the anti-human Fc antibody. LFA-1 or I235A was then injected at different concentrations, using a flow rate of 10 ml/min, and the surface plasmon resonance was recorded. After each concentration of LFA-1 or I235A was allowed to bind and dissociate, the chip was stripped of ICAM-1/LFA-1 complexes with 0.1N HCl and regenerated with fresh ICAM-1/IgG1 before the next concentration of LFA-1 or I235A was analyzed. The association and dissociation rate constants (k_a and k_d , respectively) for LFA-1 and I235A were calculated using the BIA evaluation 2.0 program and its 1:1 Langmuir binding kinetics model (Pharmacia Biosensor AB). The k_a for LFA-1 and I235A were identical and equaled $2.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. However, the k_d for LFA-1 and I235A were significantly different and equaled $1.2 \times 10^{-2} \text{ s}^{-1}$ and $1.9 \times 10^{-3} \text{ s}^{-1}$, respectively. These values corresponded to an affinity dissociation rate (K_D) of 547 nM for LFA-1, which is in close agreement with the value of 500 nM calculated by Tominaga using a similar method [Tominaga, *et al J. Immunol.*, 161: 4016-4022 (1998)]. However, the corresponding K_D of 86 nM for I235A, represents a 6-fold increase in affinity for I235A over LFA-1, which was similar to the increase observed in cell-binding assays using COS-7 cell transfectants (discussed above). These data strongly suggest that the activation of LFA-1 binding to ICAM-1 caused by the I235A mutation in the LFA-1 regulatory binding site was a result of an increase in LFA-1 affinity. Therefore, the molecular mechanism whereby the LFA-1 regulatory binding site mediates its effects on LFA-1 binding to ICAM, must be effected in large part through regulation of the affinity state of LFA-1. Based on these data, the diaryl sulfide compounds which bind to the LFA-1 regulatory binding site are predicted to inhibit adhesion to ICAM-1 by lowering the affinity of LFA-1 for ICAM through an increase in the k_d of the receptor or through stabilizing the low affinity state of LFA-1.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those

skilled in the art. Accordingly, only such limitations as appear in the appended claims should be placed on the invention.

WHAT IS CLAIMED IS:

1. A method for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising the steps of (i) measuring LFA-1 and ligand binding in the presence and absence of a test compound under conditions that allow binding of LFA-1 to the ligand, (ii) identifying as a negative regulator the test compound which decreases LFA-1 binding to the ligand and which binds LFA-1 α_L polypeptide at a site presenting a conformation that binds a diaryl sulfide or a site defined by Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹, and Ile³⁰⁶ of human LFA-1.

2. A method for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising the steps of (i) measuring LFA-1 and ligand binding in the presence and absence of a test compound under conditions that allow binding of LFA-1 to the ligand, (ii) identifying as a negative regulator the test compound which decreases LFA-1 binding to the ligand and which binds LFA-1 α_L polypeptide at a site that binds a diaryl sulfide or a site defined by Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹, Ile³⁰⁶, Leu³⁰², Tyr²⁵⁷, Leu¹³², Val²³³, Val¹³⁰, and Tyr¹⁶⁶ of human LFA-1.

3. A method for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising the steps of (i) measuring LFA-1 and ligand binding in the presence and absence of a test compound under conditions that allow binding of LFA-1 to the ligand, (ii) identifying as a negative regulator the test compound which decreases LFA-1 binding to the ligand and which binds LFA-1 α_L polypeptide at a site that binds a diaryl sulfide or a site defined by Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹, Ile³⁰⁶, Lys²⁸⁷, Leu³⁰², Ile²⁵⁷, Lys³⁰⁵, Leu¹⁶¹, Leu¹³², Val²³³, Ile²⁵⁵, Val¹³⁰, Tyr¹⁶⁶, Phe¹³⁴, Phe¹⁶⁸, Phe¹⁵³, Tyr³⁰⁷, Val³⁰⁸, Ile³⁰⁹, Thr²³¹, Glu²⁸⁴, Phe²⁸⁵, Glu³⁰¹, Met¹⁵⁴, Ile²³⁷, Ile¹⁵⁰, and Leu²⁹⁵ of human LFA-1.

4. A method for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising the steps of (i) measuring LFA-1 and ligand binding under conditions that allow binding of LFA-1 to the ligand in the presence and absence of a test compound, (ii) identifying as a negative regulator the test compound which decreases LFA-1 binding to the ligand and which competes with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide for binding to LFA-1 α_L polypeptide.

5. A screening method for identifying a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 comprising the steps of (i) contacting LFA-1 with (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide in the presence and absence of a compound, and (ii) identifying the compound as a putative negative regulator wherein the compound competes with compound (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide for binding to LFA-1 α_L polypeptide.

6. The method according to any one of claim 1 through 5 wherein the natural ligand is an ICAM.

7. The method according to claim 6 wherein the ICAM is ICAM-1 or ICAM-3.

8. The method of claim 1, 2, 3, 4, 5 or 7 wherein the negative regulator is a diaryl sulfide.

9. A pharmaceutical composition comprising a negative regulator of LFA-1 binding to a natural ligand that binds LFA-1 identified by the method of claim 1, 2, 3, 4, or 5.

10. Use of a negative regulator identified by the method of claim 8 in the production of a medicament to ameliorate pathologies arising from LFA-1 binding to a natural ligand that binds LFA-1.

11. A method for inhibiting LFA-1 binding to a natural ligand that binds LFA-1 comprising the step of contacting LFA-1 with a negative regulator compound; said negative regulator binding LFA-1 α_L polypeptide at a site selected from the group consisting of a diaryl sulfide binding conformation defined by Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹, and Ile³⁰⁶ of human LFA-1 α_L polypeptide and an LFA-1 domain that binds compound (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide.

12. A method to inhibit leukocyte adhesion to endothelial cells comprising the step of contacting said leukocyte with a negative regulator of LFA-1 binding to an ICAM that binds LFA-1, said negative regulator binding an LFA-1 regulatory site selected from the group consisting of a diaryl sulfide binding conformation defined by Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹, and Ile³⁰⁶ of human LFA-1 α_L polypeptide and an LFA-1 domain that binds compound (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide.

13. A method to ameliorate a pathology arising from LFA-1 binding to a natural ligand that binds LFA-1 comprising the step of administering to an individual in need thereof a negative regulator of LFA-1 binding to the ligand in an amount effective to inhibit LFA-1 binding to the ligand, said negative regulator binding to an LFA-1 regulatory site selected from the group consisting of a diaryl sulfide binding conformation defined by Ile²⁵⁹, Leu²⁹⁸, Ile²³⁵, Val¹⁵⁷, Leu¹⁶¹, and Ile³⁰⁶ of human LFA-1 and an LFA-1 domain that binds compound (2-isopropyl-phenyl)[2-nitro-4-(E-((4-acetylpiperazin-1-yl)carbonyl)ethenyl)phenyl]sulfide.

14. The method of claim 9, 10, 11, 12, or 13 wherein the inhibitor is a diaryl sulfide.

15. The method of claim 11 or 13 wherein the natural ligand is an ICAM.

16. The method of claim 15 wherein the ICAM is ICAM-1 or ICAM-3.

17. A mutant LFA-1 α_1 polypeptide comprising an amino acid substitution selected from the group consisting of Val¹⁵⁷-Ala, Glu²¹⁸-Ala, Thr²³¹-Ala, Lys²⁸⁰-Ala, Lys²⁹⁴-Ala, Ile²³⁵-Ala, Ile²⁵⁵-Ala, Ser²⁸³-Ala, Glu²⁸⁴-Ala, Glu³⁰¹-Ala, Ile³⁰⁶-Ala, Lys¹⁶⁰-Ala, Lys²³²-Ala, Asp²⁵³-Ala, Lys²⁸⁷-Ala, Gln³⁰³-Ala, Lys³⁰⁴-Ala, Lys³⁰⁵-Ala, and Tyr³⁰⁷-Ala of SEQ ID NO: 2.

18. A monoclonal antibody secreted by hybridoma 240Q.

SEQUENCE LISTING

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Huth, Jeff

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Gln Gly Arg Pro Gly Phe Gln Glu Cys Ile Lys Gly Asn Val Asp Leu	
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<223> Description of Artificial Sequence: primer

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52

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<210> 17

<211> 34

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<210> 18

<211> 33

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<210> 19

<211> 34

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<223> Description of Artificial Sequence: primer

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34

<210> 20

<211> 34

<212> DNA

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34

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34

<210> 22
<211> 34
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<223> Description of Artificial Sequence: primer

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<211> 33
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<223> Description of Artificial Sequence: primer

<400> 23
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33

<210> 24
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33

<210> 25
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<210> 26
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<220>
<223> Description of Artificial Sequence: primer

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<211> 2704
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<213> Homo sapiens

<220>
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<222> (1)..(2307)

<400> 30

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Met	Leu	Gly	Leu	Arg	Pro	Pro	Leu	Leu	Ala	Leu	Val	Gly	Leu	Leu	Ser	
1				5					10					15		
ctc	ggg	tgc	gtc	ctc	tct	cag	gag	tgc	acg	aag	ttc	aag	gtc	agc	agc	96
Leu	Gly	Cys	Val	Leu	Ser	Gln	Glu	Cys	Thr	Lys	Phe	Lys	Val	Ser	Ser	
			20					25					30			
tgc	cgg	gaa	tgc	atc	gag	tcg	ggg	ccc	ggc	tgc	acc	tgg	tgc	cag	aag	144
Cys	Arg	Glu	Cys	Ile	Glu	Ser	Gly	Pro	Gly	Cys	Thr	Trp	Cys	Gln	Lys	
		35					40					45				
ctg	aac	ttc	aca	ggg	ccg	ggg	gat	cct	gac	tcc	att	cgc	tgc	gac	acc	192
Leu	Asn	Phe	Thr	Gly	Pro	Gly	Asp	Pro	Asp	Ser	Ile	Arg	Cys	Asp	Thr	
	50					55					60					
cgg	cca	cag	ctg	ctc	atg	agg	ggc	tgt	gcg	gct	gac	gac	atc	atg	gac	240
Arg	Pro	Gln	Leu	Leu	Met	Arg	Gly	Cys	Ala	Ala	Asp	Asp	Ile	Met	Asp	
	65				70				75					80		
ccc	aca	agc	ctc	gct	gaa	acc	cag	gaa	gac	cac	aat	ggg	ggc	cag	aag	288
Pro	Thr	Ser	Leu	Ala	Glu	Thr	Gln	Glu	Asp	His	Asn	Gly	Gly	Gln	Lys	
				85					90					95		
cag	ctg	tcc	cca	caa	aaa	gtg	acg	ctt	tac	ctg	cga	cca	ggc	cag	gca	336
Gln	Leu	Ser	Pro	Gln	Lys	Val	Thr	Leu	Tyr	Leu	Arg	Pro	Gly	Gln	Ala	
			100					105					110			
gca	gcg	ttc	aac	gtg	acc	ttc	cgg	cgg	gcc	aag	ggc	tac	ccc	atc	gac	384
Ala	Ala	Phe	Asn	Val	Thr	Phe	Arg	Arg	Ala	Lys	Gly	Tyr	Pro	Ile	Asp	
		115					120					125				
ctg	tac	tat	ctg	atg	gac	ctc	tcc	tac	tcc	atg	ctt	gat	gac	ctc	agg	432
Leu	Tyr	Phe	Leu	Met	Asp	Leu	Ser	Tyr	Ser	Met	Leu	Asp	Asp	Leu	Arg	
	130					135					140					
aat	gtc	aag	aag	cta	ggt	ggc	gac	ctg	ctc	cgg	gcc	ctc	aac	gag	atc	480
Asn	Val	Lys	Lys	Leu	Gly	Gly	Asp	Leu	Leu	Arg	Ala	Leu	Asn	Glu	Ile	
	145				150					155				160		
acc	gag	tcc	ggc	cgc	att	ggc	ttc	ggg	tcc	ttc	gtg	gac	aag	acc	gtg	528
Thr	Glu	Ser	Gly	Arg	Ile	Gly	Phe	Gly	Ser	Phe	Val	Asp	Lys	Thr	Val	
				165				170						175		
ctg	ccg	ttc	gtg	aac	acg	cac	cct	gat	aag	ctg	cga	aac	cca	tgc	ccc	576
Leu	Pro	Phe	Val	Asn	Thr	His	Pro	Asp	Lys	Leu	Arg	Asn	Pro	Cys	Pro	
			180					185					190			
aac	aag	gag	aaa	gag	tgc	cag	ccc	ccg	ttt	gcc	ttc	agg	cac	gtg	ctg	624
Asn	Lys	Glu	Lys	Glu	Cys	Gln	Pro	Pro	Phe	Ala	Phe	Arg	His	Val	Leu	
		195					200					205				
aag	ctg	acc	aac	aac	tcc	aac	cag	ttt	cag	acc	gag	gtc	ggg	aag	cag	672
Lys	Leu	Thr	Asn	Asn	Ser	Asn	Gln	Phe	Gln	Thr	Glu	Val	Gly	Lys	Gln	
		210				215					220					
ctg	att	tcc	gga	aac	ctg	gat	gca	ccc	gag	ggt	ggg	ctg	gac	gcc	atg	720
Leu	Ile	Ser	Gly	Asn	Leu	Asp	Ala	Pro	Glu	Gly	Gly	Leu	Asp	Ala	Met	
	225				230				235					240		
atg	cag	gtc	gcc	gcc	tgc	ccg	gag	gaa	atc	ggc	tgg	cgc	aac	gtc	acg	768
Met	Gln	Val	Ala	Ala	Cys	Pro	Glu	Glu	Ile	Gly	Trp	Arg	Asn	Val	Thr	

245										250										255									
cgg	ctg	ctg	gtg	ttt	gcc	act	gat	gac	ggc	ttc	cat	ttc	gcg	ggc	gac														
Arg	Leu	Leu	Val	Phe	Ala	Thr	Asp	Asp	Gly	Phe	His	Phe	Ala	Gly	Asp														
			260					265					270																
gga	aag	ctg	ggc	gcc	atc	ctg	acc	ccc	aac	gac	ggc	cgc	tgt	cac	ctg														
Gly	Lys	Leu	Gly	Ala	Ile	Leu	Thr	Pro	Asn	Asp	Gly	Arg	Cys	His	Leu														
		275					280					285																	
gag	gac	aac	ttg	tac	aag	agg	agc	aac	gaa	ttc	gac	tac	cca	tcg	gtg														
Glu	Asp	Asn	Leu	Tyr	Lys	Arg	Ser	Asn	Glu	Phe	Asp	Tyr	Pro	Ser	Val														
	290					295					300																		
ggc	cag	ctg	gcg	cac	aag	ctg	gct	gaa	aac	aac	atc	cag	ccc	atc	ttc														
Gly	Gln	Leu	Ala	His	Lys	Leu	Ala	Glu	Asn	Asn	Ile	Gln	Pro	Ile	Phe														
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Ala	Val	Thr	Ser	Arg	Met	Val	Lys	Thr	Tyr	Glu	Lys	Leu	Thr	Glu	Ile														
				325					330				335																
atc	ccc	aag	tca	gcc	gtg	ggg	gag	ctg	tct	gag	gac	tcc	agc	aat	gtg														
Ile	Pro	Lys	Ser	Ala	Val	Gly	Glu	Leu	Ser	Glu	Asp	Ser	Ser	Asn	Val														
			340				345					350																	
gtc	cat	ctc	att	aag	aat	gct	tac	aat	aaa	ctc	tcc	tcc	agg	gtc	ttc														
Val	His	Leu	Ile	Lys	Asn	Ala	Tyr	Asn	Lys	Leu	Ser	Ser	Arg	Val	Phe														
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ctg	gat	cac	aac	gcc	ctc	ccc	gac	acc	ctg	aaa	gtc	acc	tac	gac	tcc														
Leu	Asp	His	Asn	Ala	Leu	Pro	Asp	Thr	Leu	Lys	Val	Thr	Tyr	Asp	Ser														
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ttc	tgc	agc	aat	gga	gtg	acg	cac	agg	aac	cag	ccc	aga	ggt	gac	tgt														
Phe	Cys	Ser	Asn	Gly	Val	Thr	His	Arg	Asn	Gln	Pro	Arg	Gly	Asp	Cys														
385					390				395				400																
gat	ggc	gtg	cag	atc	aat	gtc	ccg	atc	acc	ttc	cag	gtg	aag	gtc	acg														
Asp	Gly	Val	Gln	Ile	Asn	Val	Pro	Ile	Thr	Phe	Gln	Val	Lys	Val	Thr														
				405				410				415																	
gcc	aca	gag	tgc	atc	cag	gag	cag	tcg	ttt	gtc	atc	cgg	gcg	ctg	ggc														
Ala	Thr	Glu	Cys	Ile	Gln	Glu	Gln	Ser	Phe	Val	Ile	Arg	Ala	Leu	Gly														
			420					425				430																	
ttc	acg	gac	ata	gtg	acc	gtg	cag	gtt	ctt	ccc	cag	tgt	gag	tgc	cgg														
Phe	Thr	Asp	Ile	Val	Thr	Val	Gln	Val	Leu	Pro	Gln	Cys	Glu	Cys	Arg														
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tgc	cgg	gac	cag	agc	aga	gac	cgc	agc	ctc	tgc	cat	ggc	aag	ggc	ttc														
Cys	Arg	Asp	Gln	Ser	Arg	Asp	Arg	Ser	Leu	Cys	His	Gly	Lys	Gly	Phe														
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ttg	gag	tgc	ggc	atc	tgc	agg	tgt	gac	act	ggc	tac	att	ggg	aaa	aac														
Leu	Glu	Cys	Gly	Ile	Cys	Arg	Cys	Asp	Thr	Gly	Tyr	Ile	Gly	Lys	Asn														
465					470				475				480																
tgt	gag	tgc	cag	aca	cag	ggc	cgg	agc	agc	cag	gag	ctg	gaa	gga	agc														
Cys	Glu	Cys	Gln	Thr	Gln	Gly	Arg	Ser	Ser	Gln	Glu	Leu	Glu	Gly	Ser														
				485				490					495																

tgc cgg aag gac aac aac tcc atc atc tgc tca ggg ctg ggg gac tgt	1536
Cys Arg Lys Asp Asn Asn Ser Ile Ile Cys Ser Gly Leu Gly Asp Cys	
500 505 510	
gtc tgc ggg cag tgc ctg tgc cac acc agc gac gtc ccc ggc aag ctg	1584
Val Cys Gly Gln Cys Leu Cys His Thr Ser Asp Val Pro Gly Lys Leu	
515 520 525	
ata tac ggg cag tac tgc gag tgt gac acc atc aac tgt gag cgc tac	1632
Ile Tyr Gly Gln Tyr Cys Glu Cys Asp Thr Ile Asn Cys Glu Arg Tyr	
530 535 540	
aac ggc cag gtc tgc ggc ggc ccg ggg agg ggg ctc tgc ttc tgc ggg	1680
Asn Gly Gln Val Cys Gly Gly Pro Gly Arg Gly Leu Cys Phe Cys Gly	
545 550 555 560	
aag tgc cgc tgc cac ccg ggc ttt gag ggc tca gcg tgc cag tgc gag	1728
Lys Cys Arg Cys His Pro Gly Phe Glu Gly Ser Ala Cys Gln Cys Glu	
565 570 575	
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Arg Thr Thr Glu Gly Cys Leu Asn Pro Arg Arg Val Glu Cys Ser Gly	
580 585 590	
cgt ggc cgg tgc cgc tgc aac gta tgc gag tgc cat tca ggc tac cag	1824
Arg Gly Arg Cys Arg Cys Asn Val Cys Glu Cys His Ser Gly Tyr Gln	
595 600 605	
ctg cct ctg tgc cag gag tgc ccc ggc tgc ccc tca ccc tgt ggc aag	1872
Leu Pro Leu Cys Gln Glu Cys Pro Gly Cys Pro Ser Pro Cys Gly Lys	
610 615 620	
tac atc tcc tgc gcc gag tgc ctg aag ttc gaa aag ggc ccc ttt ggg	1920
Tyr Ile Ser Cys Ala Glu Cys Leu Lys Phe Glu Lys Gly Pro Phe Gly	
625 630 635 640	
aag aac tgc agc gcg gcg tgt ccg ggc ctg cag ctg tcg aac aac ccc	1968
Lys Asn Cys Ser Ala Ala Cys Pro Gly Leu Gln Leu Ser Asn Asn Pro	
645 650 655	
gtg aag ggc agg acc tgc aag gag agg gac tca gag ggc tgc tgg gtg	2016
Val Lys Gly Arg Thr Cys Lys Glu Arg Asp Ser Glu Gly Cys Trp Val	
660 665 670	
gcc tac acg ctg gag cag cag gac ggg atg gac cgc tac ctc atc tat	2064
Ala Tyr Thr Leu Glu Gln Gln Asp Gly Met Asp Arg Tyr Leu Ile Tyr	
675 680 685	
gtg gat gag agc cga gag tgt gtg gca ggc ccc aac atc gcc gcc atc	2112
Val Asp Glu Ser Arg Glu Cys Val Ala Gly Pro Asn Ile Ala Ala Ile	
690 695 700	
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Val Gly Gly Thr Val Ala Gly Ile Val Leu Ile Gly Ile Leu Leu Leu	
705 710 715 720	
gtc atc tgg aag gct ctg atc cac ctg agc gac ctc cgg gag tac agg	2208
Val Ile Trp Lys Ala Leu Ile His Leu Ser Asp Leu Arg Glu Tyr Arg	
725 730 735	
cgc ttt gag aag gag aag ctc aag tcc cag tgg aac aat gat aat ccc	2256
Arg Phe Glu Lys Glu Lys Leu Lys Ser Gln Trp Asn Asn Asp Asn Pro	

740 745 750
 ctt ttc aag agc gcc acc acg acg gtc atg aac ccc aag ttt gct gag 2304
 Leu Phe Lys Ser Ala Thr Thr Thr Val Met Asn Pro Lys Phe Ala Glu
 755 760 765
 agt taggagcact tggatgaagac aaggccgtca ggaccaccca tgtctgcccc 2357
 Ser
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 <213> Homo sapiens

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 35 40 45
 Leu Asn Phe Thr Gly Pro Gly Asp Pro Asp Ser Ile Arg Cys Asp Thr
 50 55 60
 Arg Pro Gln Leu Leu Met Arg Gly Cys Ala Ala Asp Asp Ile Met Asp
 65 70 75 80
 Pro Thr Ser Leu Ala Glu Thr Gln Glu Asp His Asn Gly Gly Gln Lys
 85 90 95
 Gln Leu Ser Pro Gln Lys Val Thr Leu Tyr Leu Arg Pro Gly Gln Ala
 100 105 110
 Ala Ala Phe Asn Val Thr Phe Arg Arg Ala Lys Gly Tyr Pro Ile Asp
 115 120 125
 Leu Tyr Tyr Leu Met Asp Leu Ser Tyr Ser Met Leu Asp Asp Leu Arg
 130 135 140
 Asn Val Lys Lys Leu Gly Gly Asp Leu Leu Arg Ala Leu Asn Glu Ile
 145 150 155 160
 Thr Glu Ser Gly Arg Ile Gly Phe Gly Ser Phe Val Asp Lys Thr Val
 165 170 175
 Leu Pro Phe Val Asn Thr His Pro Asp Lys Leu Arg Asn Pro Cys Pro

Val Cys Gly Gln Cys Leu Cys His Thr Ser Asp Val Pro Gly Lys Leu
 515 520 525
 Ile Tyr Gly Gln Tyr Cys Glu Cys Asp Thr Ile Asn Cys Glu Arg Tyr
 530 535 540
 Asn Gly Gln Val Cys Gly Gly Pro Gly Arg Gly Leu Cys Phe Cys Gly
 545 550 555 560
 Lys Cys Arg Cys His Pro Gly Phe Glu Gly Ser Ala Cys Gln Cys Glu
 565 570 575
 Arg Thr Thr Glu Gly Cys Leu Asn Pro Arg Arg Val Glu Cys Ser Gly
 580 585 590
 Arg Gly Arg Cys Arg Cys Asn Val Cys Glu Cys His Ser Gly Tyr Gln
 595 600 605
 Leu Pro Leu Cys Gln Glu Cys Pro Gly Cys Pro Ser Pro Cys Gly Lys
 610 615 620
 Tyr Ile Ser Cys Ala Glu Cys Leu Lys Phe Glu Lys Gly Pro Phe Gly
 625 630 635 640
 Lys Asn Cys Ser Ala Ala Cys Pro Gly Leu Gln Leu Ser Asn Asn Pro
 645 650 655
 Val Lys Gly Arg Thr Cys Lys Glu Arg Asp Ser Glu Gly Cys Trp Val
 660 665 670
 Ala Tyr Thr Leu Glu Gln Gln Asp Gly Met Asp Arg Tyr Leu Ile Tyr
 675 680 685
 Val Asp Glu Ser Arg Glu Cys Val Ala Gly Pro Asn Ile Ala Ala Ile
 690 695 700
 Val Gly Gly Thr Val Ala Gly Ile Val Leu Ile Gly Ile Leu Leu Leu
 705 710 715 720
 Val Ile Trp Lys Ala Leu Ile His Leu Ser Asp Leu Arg Glu Tyr Arg
 725 730 735
 Arg Phe Glu Lys Glu Lys Leu Lys Ser Gln Trp Asn Asn Asp Asn Pro
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 Leu Phe Lys Ser Ala Thr Thr Thr Val Met Asn Pro Lys Phe Ala Glu
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Ser

<210> 32

<211> 47

<212> PRT

<213> Homo sapiens

<400> 32

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Ser Ala Gln Leu Glu Lys Glu Leu Gln Ala Leu Glu Lys Glu Asn Ala

20

25

30

Gln Leu Glu Trp Glu Leu Gln Ala Leu Glu Lys Glu Leu Ala Gln
35 40 45

<210> 33

<211> 47

<212> PRT

<213> Homo sapiens

<400> 33

Thr Arg Ser Ser Ala Asp Leu Val Pro Arg Gly Ser Thr Thr Ala Pro
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20 25 30

Gln Leu Lys Trp Lys Leu Gln Ala Leu Lys Lys Lys Leu Ala Gln
35 40 45